Resistance to Macrolide Antibiotics in Public Health Pathogens

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Macrolide resistance mechanisms can be target-based with a change in a 23S ribosomal RNA (rRNA) residue or a mutation in ribosomal protein L4 or L22 affecting the ribosome's interaction with the antibiotic. Alternatively, mono- or dimethylation of A2058 in domain V of the 23S rRNA by an acquired rRNA methyltransferase, the product of an *erm* (erythromycin ribosome methylation) gene, can interfere with antibiotic binding. Acquired genes encoding efflux pumps, most predominantly *mef*(A) + *msr*(D) in pneumococci/streptococci and *msr*(A/B) in staphylococci, also mediate resistance. Drug-inactivating mechanisms include phosphorylation of the 2′-hydroxyl of the amino sugar found at position C5 by phosphotransferases and hydrolysis of the macrocyclic lactone by esterases. These acquired genes are regulated by either translation or transcription attenuation, largely because cells are less fit when these genes, especially the rRNA methyltransferases, are highly induced or constitutively expressed. The induction of gene expression is cleverly tied to the mechanism of action of macrolides, relying on antibiotic-bound ribosomes stalled at specific sequences of nascent polypeptides to promote transcription or translation of downstream sequences.

Macrolide antibiotics are polyketides composed of a 14-, 15-, or 16-membered macrocyclic lactone ring (14-, 15-, and 16-membered) to which several sugars and/or side chains have been attached by the producing organism or as modifications during semisynthesis in the laboratory (Figs. 1 and 2). Newer semisynthetic derivations, like ketolides telithromycin, and solithromycin, have a C3-keto group in place of the C3 cladinose (akin to naturally occurring pikromycin) (Brockmann and Henkel 1950) and an 11,12-cyclic carbamate with an extended alkyl–aryl side chain that increases the affinity of the antibiotic for the ribosome by 10- to 100-fold (Hansen et al. 1999; Dunkle et al. 2010); in the case of solithromycin, a fluorine substituent at C2 provides an additional ribosomal interaction (Llano-Sotelo et al. 2010). Macrolides continue to be important in the therapeutic treatment of community-acquired pneumonia (*Streptococcus pneumoniae*, *Haemophilus influenzae*, *Moraxella catarrhalis*, and atypicals *Legionella pneumophila*, *Mycoplasma pneumoniae*, *Chlamydia pneumoniae*), sexually transmitted diseases (*Neiserria gonorhoeae*, *Chlamydia trachomatis*, *Mycoplasma genitalium*), shigellosis, and salmonellosis. With solithromycin heading for a new drug application (NDA) filing in 2016 and having the in vitro potency to treat erythromycin-resistant

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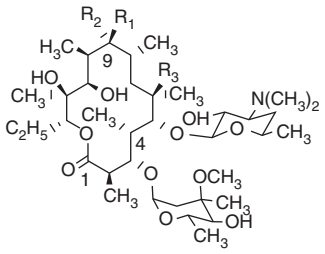
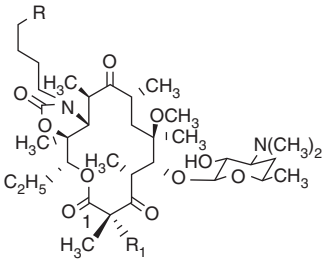
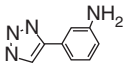
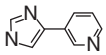
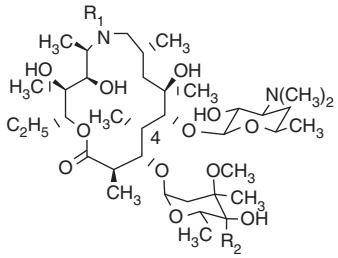


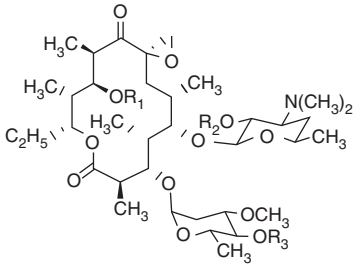
	R_1	R_2	R_3
Erythromycin	O	O	OH
Clarithromycin	O	O	OCH_3
Roxithromycin	$NOCH_2OC_2H_4OCH_3$		OH



	R	R_1
Solithromycin		F
Telithromycin		H



	R_1	R_2
Azithromycin	CH_3	H
Tulathromycin	H	$CH_2NH(CH_2)_2CH_3$



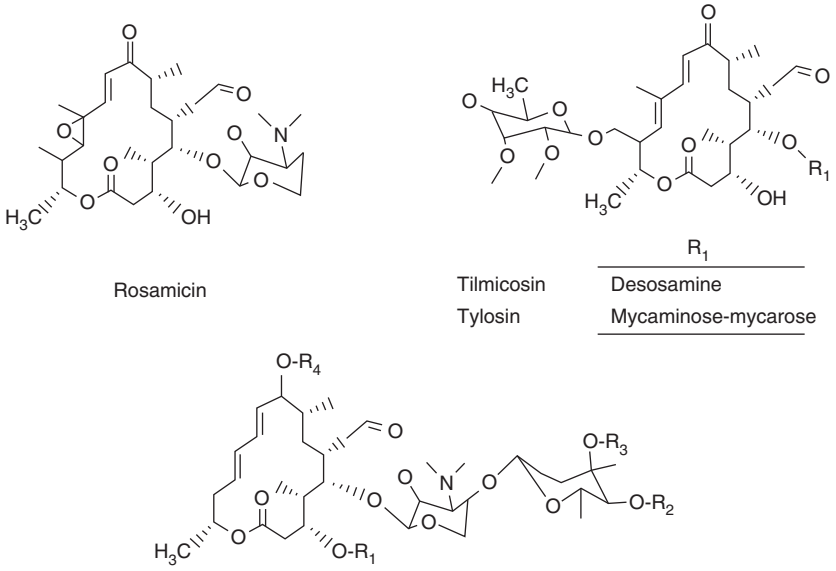
	R_1, R_2, R_3
Oleandomycin	H
Troleandomycin	$COCH_3$

Figure 1. Structures of 14- and 15-membered macrolides.

pneumococci and gonococci (Farrell et al. 2015; Hook et al. 2015), macrolides/ketolides will continue as an important part of the antibiotic armamentarium.

The mechanism of action of macrolides has been further refined through a combination of genetic, biochemical, crystallographic, and ribosome profiling studies (Tu et al. 2005; Dunkle et al. 2010; Kannan et al. 2012, 2014; Gupta et al. 2016). Macrolides/ketolides are sensed by the ribosome and, in the presence of certain macrolide-stalling nascent amino acid chain-dependent motifs, selectively inhibit protein synthesis. Further, and to different extents, ketolides and macrolides cause frameshifting, leading to aberrant protein synthesis.

Shortly after its clinical debut in 1953, resistance to erythromycin in staphylococci was described and was likely mediated by methylation of the 23S ribosomal RNA (rRNA) at nucleotide A2058 (*Escherichia coli* numbering) encoded by an erythromycin ribosomal methyltransferase (*erm*) gene (Weisblum 1995a). Erm methyltransferases add one or two methyl groups to the N-6 exocyclic amino group of A2058, disrupting the key hydrogen bond between A2058 and the desosamine sugar at C5 (Fig. 3). Ribosomal methylation by methyltransferases encoded by *erm* genes remains the most widespread macrolide resistance in pathogenic bacteria, with certain *erm* genes more predominantly found in some species. Streptococci



Rosamicin

	R_1
Tilmicosin	Desosamine
Tylosin	Mycaminose-mycarose

	R_1	R_2	R_3	R_4
Spiramycin	H	H	H	Forosamine
Josamycin	$COCH_3$	H	$COCH_2CH(CH_3)_2$	H
Kitasamycin	CH_3	H	H	H
Midecamycin	Propionyl	Propionyl	H	H

Figure 2. Structures of 16-membered macrolides.

generally have *erm*(B) or *erm*(A), subclass *erm*(TR), whereas *erm*(A), *erm*(B), or *erm*(C) are found in staphylococci and *erm*(F) in anaerobes and *H. influenzae* (Table 2, and references therein; see also faculty.washington.edu/marilynr). There can be either mono- or dimethylation of A2058 and the degree of rRNA dimethylation can determine ketolide resistance (Douthwaite et al. 2005). Most *erm* genes are inducible by 14- and 15-membered macrolides, whereby translation repression of the *erm* methyltransferase gene, because of the sequestration of its ribosome-binding site (RBS) by messenger RNA (mRNA) secondary structure, is relieved by binding of the inducer to the ribosome (Horinouchi and Weisblum 1980; Depardieu et al. 2007; Subramaniam et al. 2011). Upstream of the start codon of a methyltransferase gene is an open reading frame (ORF) that produces leader peptides of different lengths (8–38 amino acids), each containing a macrolide stalling motif; when the macrolide-bound ribosome pauses, the attenuator, a stem and loop structure that encompasses the RBS, is disrupted, resulting in ribosomal binding and synthesis of the methyltransferase (Subramaniam et al. 2011; Arenz et al. 2014a,b). Although most *erm* genes are regulated by translation attenuation, a few genes (e.g., *erm*(K)) are regulated by transcription attenuation (Kwak et al. 1991; Choi et al. 1997) or through inducible transcription factors (Morris et al. 2005). Ketolide induction has been described for *erm*(C) and involves promotion of frameshifting in the *erm*(C) leader (*ermCL*) mRNA, leading to bypass of the *ermCL* stop codon, via rearrangement of the secondary mRNA structure, allowing expression of the downstream resistance gene (Gupta et al. 2013a).

There are two families of macrolide efflux pumps with regulation that is at least in part, transcriptionally mediated—*mef*, a major-facil-

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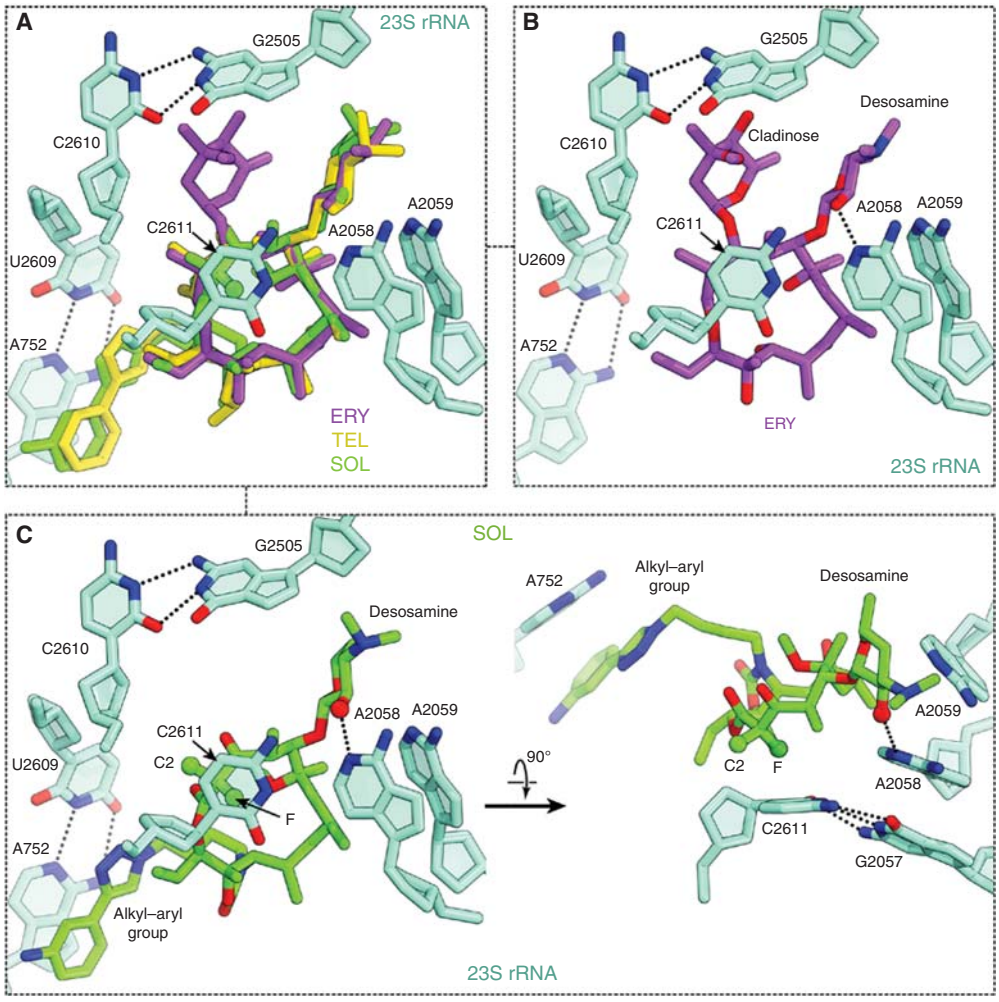

Figure 3. A model based on the crystal structure of the 70S *Escherichia coli* ribosome bound to erythromycin (PDB ID codes 3OFO, 3OFP, 3OFR, 3OFQ), telithromycin (PDB ID codes 3OAQ, 3OAR, 3OAS, 3OAT), and solithromycin (PDB ID code 4WWW) (Dunkle et al. 2010; Llano-Sotelo et al. 2010). (*A*) A comparison of the conformations of erythromycin (ERY, magenta), telithromycin (TEL, gold), and solithromycin (SOL, green) in their binding sites at the top of the nascent peptide exit tunnel (PET) comprised of 23S ribosomal RNA (rRNA). 23S rRNA residues are marked, with nitrogen in dark blue and oxygen in red. Hydrogen bonds are indicated between residues by dotted lines, including between residues U2609 in domain V and A752 in domain II of 23S rRNA. The alkyl–aryl arm of telithromycin and solithromycin is shown stacking with A752. (*B*) Erythromycin-only view. The key hydrogen bond between the 2′ hydroxyl of the desosamine and the N1 of A2058 is indicated. The exocyclic N6 amino group that is methylated by Erm methyltransferases is notable next to the N1 of A2058. (*C*) Solithromycin-only view. The *left* side of the figure displays solithromycin in the same conformation as macrolides in *A* and *B*. The C2-F is visible through the ring of C2611, but a better view of its interaction with C2611 is displayed when the view is rotated by 90°, with the C2-F stacking with the hydrophobic side of C2611. C2611 is paired through three hydrogen bonds to G2057.

itator-superfamily pump that confers resistance to most 14- and 15-membered macrolides (Leclercq and Courvalin 2002; Sutcliffe and Leclercq 2002; Chancey et al. 2011) and *msr*, a member of the ATP-binding cassette (ABC) superfamily that generally confers resistance to 14- and 15-membered macrolides and streptogramin B and low-level resistance to ketolides (Sutcliffe and Leclercq 2002; Chancey et al. 2011).

Intrinsic efflux pumps that are not specific to macrolides exist in different species. These pumps are often responsible for limiting macrolide spectrum in Gram-negative species and overexpression of multidrug efflux pumps is associated with clinically relevant drug resistance in both Gram-negative and Gram-positive species. Interested readers are referred to recent reviews (Costa et al. 2013; Blair et al. 2014; Delmar et al. 2014; Sun et al. 2014).

Mutations in 23S rRNA, L4, and/or L22 ribosomal proteins can confer macrolide resistance because the mutation is technically in the 23S rRNA gene. In addition, macrolides can be inactivated by esterases or phosphotransferases (in public health pathogens and macrolide producers) or by glycosyltransferases (decribed in many strains of *Streptomyces* producing polyketides or polyether antibiotics; *Micromonospora purpurea*; *Nocardia asteroides*), deacylases (*N. asteroides*), or formyl reductases (*N. asteroides*, *Nocardia brasiliensis*, *Nocardia otitidiscaviarum*) (Sutcliffe and Leclercq 2002; Roberts 2008; Shakya and Wright 2010; Morar et al. 2012). Many strains carry more than one macrolide resistance mechanism, sometimes on the same mobile element.

This review will focus on antimicrobial resistance mechanisms to macrolides primarily in public health pathogens. Recent reviews on the mechanisms of macrolide resistance are recommended (Leclercq and Courvalin 2002; Sutcliffe and Leclercq 2002; Franceschi et al. 2004; Depardieu et al. 2007; Roberts 2008; Kannan and Mankin 2011; Wilson 2014) as well as the website for macrolide–lincosamide–streptogramin resistances maintained by Marilyn Roberts (see faculty.washington.edu/marilynr). Based on the paper published in 1999 that set out to prevent duplicate genes being renamed when discovered in a new species or as part of a novel mobile element (Roberts et al. 1999), macrolide-resistant genes are considered in the same family if they have $\geq$80% amino acid identity from the original gene identified for that family.

MACROLIDE MECHANISM OF ACTION

All members of the macrolide class inhibit bacterial protein synthesis by binding to the 23S rRNA in the large ribosomal subunit (50S) downstream from the peptidyltransferase center (PTC), the catalytic site for peptide bond formation (for overview of protein synthesis, see Arenz and Wilson 2016) (Wilson 2009, 2014; Dunkle et al. 2010; Kannan et al. 2014). Macrolides/ketolides bind at the entrance of the peptide exit tunnel (PET) just above the constriction formed by extended loops of ribosomal proteins L4 and L22 (Yusupov et al. 2001; Davydova et al. 2002; Hansen et al. 2002; Schlunzen et al. 2003; Tu et al. 2005; Dunkle et al. 2010), further restricting the effective diameter of the PET. The macrocyclic lactone and the C5 sugars overlap (Fig. 3A). The sugar at C5 (often desosamine) is positioned toward the PTC, and macrolides like tylosin that have a disaccharide at the C5 position, reach deeper into the PTC. The 2′ hydroxyl of desosamine sugar at C5 makes a key hydrogen bond contact with the N1 atom of A2058 and modification at this position by either mutation or methylation of the N6 exocyclic amine results in macrolide resistance (see Fig. 3B) (Sutcliffe and Leclercq 2002; Franceschi et al. 2004; Tu et al. 2005; Dunkle et al. 2010). Other residues help define a local binding conformation for macrolides, including G2057 and C2611 that form a Watson–Crick base pair with each other and to which the hydrophobic face of the lactone ring is packed (seen best in Fig. 3C). For ketolides, telithromycin, and solithromycin, the extended alkyl–aryl arm of each drug is oriented down the tunnel and makes a stacking interaction with a base pair formed by A752 and U2609 in the 23S rRNA (Fig. 3A,C); these side chains align closely in the crystal structure of each drug complexed to *E. coli* 70S ribosome, but are positioned differently from the crystal structures

of telithromycin complexed with either *Deinococcus radiodurans* or *Haloarcula marismortui* (Schlunzen et al. 2003; Tu et al. 2005; Dunkle et al. 2010; Llano-Sotelo et al. 2010), likely a result of the absence of the A752-U2609 base pair seen in most pathogenic bacteria. Solithromycin has an additional stacking interaction with the hydrophobic portion of C2611 via its C2-fluorine, thereby conferring eightfold better activity against *S. pneumoniae* constitutive *erm*(B) isolates than a corresponding structure with hydrogen at C2 (Fig. 3C) (Llano-Sotelo et al. 2010).

Until recently, macrolides were thought to inhibit protein synthesis by sterically blocking nascent peptides as they transversed the PET (Hansen et al. 2002; Voss et al. 2006). However, despite the constriction formed from L4 and L22 loops and bound macrolide, there is still room in the PET for nascent, unfolded peptides to successfully negotiate the tunnel (Tu et al. 2005; Dunkle et al. 2010; Kannan et al. 2012). Further, genome-wide ribosome profiling analyses in *E. coli* have shown that ribosomes with bound erythromycin or telithromycin allow a compound-dependent subset of proteins to be synthesized, rather than act as general translation inhibitors (Kannan et al. 2012, 2014). Further work has shown that ribosomes bound with a macrolide or ketolide are impaired in the efficient catalysis of peptide bond formation and that this impairment is sequence- and context-specific (i.e., dependent on macrolide stalling motifs) (Arenz et al. 2014a; Kannan et al. 2014; Sothiselvam et al. 2014). If a macrolide-stalling motif is encountered near the amino terminus of an ORF, then ribosomal stalling of a short nascent peptide likely leads to premature release of peptidyl-tRNA, consistent with early biochemical studies with erythromycin (Otaka and Kaji 1975; Menninger 1985; Tenson et al. 2003). However, macrolide stalling motifs can be hundreds of codons away from the start codon, resulting in the synthesis of large peptides and, for some proteins that have no translation arrest sequences, synthesis of full length proteins (Kannan et al. 2012). Translation arrest can occur because specific sequences (macrolide stalling motifs) of the nascent leader peptide in the ribosomal tunnel sense the ribosome-bound antibiotic and, through interactions with it and with elements of the tunnel wall, induce conformational rearrangements that are communicated to the PTC so as to stop translation (Arenz et al. 2014a,b; Sothiselvam et al. 2014). Alternatively, macrolide- and peptide-dependent programmed translation arrest is also defined by the nature of the amino acid residues in the PTC (Kannan et al. 2014). Amazingly, the "nose" of the ribosome can sense small structural changes in the macrolide as well as discriminate a single amino acid difference in the nascent peptide (Gupta et al. 2016). Further, the discriminating properties of the PET allow for regulation of *cis*-located target gene expression, protein targeting and folding, and response to additional cellular factors (Ito et al. 2010; Kannan et al. 2014; Gupta et al. 2016).

Investigation into the mechanism by which ketolides induce *erm*(C) uncovered another mechanism of action of ketolides and macrolides—promotion of frameshifting (Gupta et al. 2013a). Intriguingly, the extent of reduction in translational fidelity is compound-dependent and is reliant on the antibiotic allosterically influencing the reading-frame maintenance in the 30S ribosomal subunit, some 90 Å away from the macrolide/ketolide binding site in the 50S ribosomal subunit (Gupta et al. 2013a). Because 25% of the entire *E. coli* proteome continues to be synthesized in the presence of telithromycin (Kannan et al. 2012), production of aberrant cellular proteins may also be important to its antibacterial action.

MACROLIDE-RESISTANT MECHANISMS

Ribosomal Modifications

23S rRNA Mutations

Mutants that are resistant to one or more of the MLS_B antibiotics, because of base substitutions in either domain V or helix 35 in domain II of 23S rRNA or in ribosomal proteins L4 or L22, provide genetic evidence that these antibiotics interact with the ribosome (Figs. 3 and 4) (Vester and Douthwaite 2001; Sutcliffe and Leclercq 2002; Franceschi et al. 2004). Macrolides primarily interact with A2058 and A2059 of the

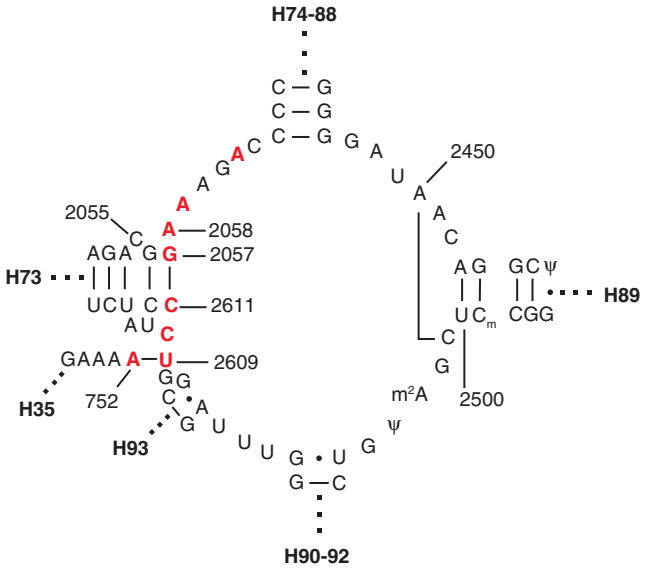

Figure 4. The secondary structure of the 3′ region of 23S ribosomal RNA (rRNA) domain V. Nucleotides in red indicate mutations that can yield 14/15-membered macrolide, 16-membered macrolide, and/or ketolide resistance (see Schwarz et al. 2016 for mutations in this region that alter lincosamides, streptogramins, phenicols, and pleuromutilins). rRNA helices that stem from this region are designated with dotted lines. Residue A752 is in the hairpin 35 loop of domain II.

23S rRNA and mutations in these nucleotides have been found in many macrolide-resistant bacterial strains, generally in pathogens (*Mycobacterium*, *Brachyspira*, *Helicobacter*, *Treponema*) with just one or two copies of *rrl*, the gene that codes for 23S rRNA or, in pathogens with three or more rRNA genes, may develop during chronic treatment of macrolides (Table 1). However, mutations in these positions as well as at G2057 in combination with A2059, and at C2611, have been found in clinical isolates and laboratory mutants of *S. pneumoniae* (Tait-Kamradt et al. 2000a,b; Canu et al. 2002), at A2058 and C2611 in clinical isolates of *Streptococcus pyogenes* (Malbruny et al. 2002; Jalava et al. 2004), and at A2058, A2059, or both A2059 and G2160 in clinical isolates of *H. influenzae* (Peric et al. 2003). Consistently, mutations at A2058 and A2059 are the most frequently observed and have a strong phenotype in all species, generally conferring macrolide–lincosamide–streptogramin B-ketolide (MLS_BK) resistance in most isolates. A comprehensive listing of ribosomal 23S rRNA mutations isolated in *S. pneumoniae*, *S. pyogenes*, and *H. influenzae* before 2005 has been assembled by Franceschi et al. (2004). Table 1 updates the base substitutions and extends the citings to other species.

With the increase in use of the macrolide azithromycin as a maintenance treatment for cystic fibrosis (CF) patients, there has been an increase in the levels of MLS_B-resistant *Staphylococcus aureus* isolated from CF patients. Six azithromycin- and erythromycin-resistant isolates of *S. aureus* from CF patients after treatment with azithromycin (Prunier et al. 2002), that did not carry resistance determinant *erm* or *msr*(A) genes, were found to carry mutations A2058G, A2058T, or A2059G with copy numbers of mutant alleles ranging from three of five and four of five to four of six *rrl* genes (*S. aureus* can have five or six *rrl* genes). A more recent characterization of *S. aureus* strains isolated from Czech CF patients showed high rates (29%) of strains with ribosomal mutations conferring resistance to MLS_B antibiotics with the majority in 23S rRNA (23%) (Tkadlec et al. 2015).

Table 1. Mutations in 23S rRNA (*E. coli* numbering) conferring macrolide/ketolide resistance

Nucleotide	Organism	Wild-type	Mutant	References
752	*Mycoplasma genitalium*	A	C	Jensen et al. 2014
	Streptococcus pneumoniae	A	Deletion	Canu et al. 2000
754	*Escherichia coli*	U	A	Xiong et al. 1999
2038	*M. genitalium*	C	T	Chrisment et al. 2012
2057	*E. coli*	G	A	Ettayebi et al. 1985
	Mycoplasma fermentans	G	A	Pereyre et al. 2002
	Mycoplasma hominis	G	A	Pereyre et al. 2002
	Propionibacterium spp.	G	A	Ross et al. 1997
2057 + 2032	*E. coli*	G/G	A/A	Douthwaite 1992
2057 + 2032	*Helicobacter pylori*	A/G	G/A	Hulten et al. 1997
2057 + 2059	*S. pneumoniae*	G/A	A/C	Fu et al. 2000
	S. pneumoniae	G/A	A/G	Farrell et al. 2004
	Streptococcus pyogenes	G/A	A/G	Doktor et al. 2001
2058 + 2160	*Haemophilus influenzae*	A/G	G/T	Peric et al. 2003
2058 + 2166	*Streptococcus pyogenes*	A/U	G/C	Farrell et al. 2006
2058 + 2160	*Haemophilus influenzae*	A/G	G/U	Peric et al. 2003
2059 + 2059	*S. pneumoniae*	A/A	G/C	Farrell et al. 2004
2058	*Brachyspira hyodysenteriae*	A	G, T	Karlsson et al. 1999
	E. coli	A	G	Vester and Garrett 1987; Douthwaite 1992
	E. coli	A	T	Sigmund et al. 1984
	H. influenzae	A	G	Clark et al. 2002
	H. pylori	A	C, G	Stone et al. 1996; Hulten et al. 1997; Occhialini et al. 1997; Versalovic et al. 1997; Debets-Ossenkopp et al. 1998; Wang and Taylor 1998
	Moraxella catarrhalis	A	T	Saito et al. 2012; Iwata et al. 2015
	Mycobacterium abscessus	A	G	Wallace et al. 1996
	Mycobacterium avium	A	C, G, T	Nash and Inderlied 1995
	Mycobacterium chelonae	A	C, G	Wallace et al. 1996
	Mycobacterium intracellulare	A	C, G, T	Meier et al. 1994
	Mycobacterium kansasii	A	T	Burman et al. 1998
	Mycobacterium smegmatis	A	G	Sander et al. 1997
	M. genitalium	A	C, G	Jensen et al. 2008; Ito et al. 2011; Chrisment et al. 2012; Gesink et al. 2012; Twin et al. 2012; Touati et al. 2014; Bissessor et al. 2015
	M. hominis	A	G	Pereyre et al. 2002
	Mycoplasma pneumoniae	A	C, G, T	Lucier et al. 1995; Matsuoka et al. 2004; Liu et al. 2009b; Peuchant et al. 2009; Xin et al. 2009; Cao et al. 2010; Kawai et al. 2013; Ye et al. 2013; Zhou et al. 2015
	Propionibacterium spp.	A	G	Ross et al. 1997
	Staphylococcus aureus	A	G, T	Prunier et al. 2002
	S. pneumoniae	A	G, T	Tait-Kamradt et al. 2000a,b; Canu et al. 2002; Farrell et al. 2004
	S. pyogenes	A	G	Jalava et al. 2004

Continued

Table 1. *Continued*

Nucleotide	Organism	Wild-type	Mutant	References
	Treponema pallidum	A	G	Stamm and Bergen 2000
2059	*H. influenzae*	A	G	Clark et al. 2002
	H. pylori	A	C, G, T	Hulten et al. 1997; Occhialini et al. 1997; Versalovic et al. 1997; Debets-Ossenkopp et al. 1998; Wang and Taylor 1998
	Propionibacterium spp.	A	G	Ross et al. 1997
	M. abscessus	A	C, G	Wallace et al. 1996
	M. avium	A	C	Nash and Inderlied 1995
	M. chelonae	A	G	Wallace et al. 1996
	M. intracellulare	A	C	Meier et al. 1994
	M. genitalium	A	C, G	Jensen et al. 2008; Ito et al. 2011; Chrisment et al. 2012; Gesink et al. 2012; Twin et al. 2012; Touati et al. 2014; Bissessor et al. 2015
	M. pneumoniae	A	G	Matsuoka et al. 2004; Peuchant et al. 2009; Xin et al. 2009; Cao et al. 2010; Kawai et al. 2013
	S. aureus	A	G	Prunier et al. 2002
	S. pneumoniae	A	C, G	Tait-Kamradt et al. 2000a; Farrell et al. 2004; Rantala et al. 2005
2062	*M. genitalium*	A	T	Chrisment et al. 2012
	M. pneumoniae	A	G	Bebear and Pereyre 2005; Peuchant et al. 2009
	S. pneumoniae	A	C	Depardieu and Courvalin 2001
2098	*H. pylori*	T	C	Kim et al. 2008; Rimbara et al. 2008[a]
2160	*H. influenzae*	G	U	Peric et al. 2003
2160–2162	*H. influenzae*	GGA	UAU	Peric et al. 2003
2164	*H. influenzae*	C	G	Peric et al. 2003
2185	*M. genitalium*	T	G	Shimada et al. 2011
2609	*E. coli*	U	C	Garza-Ramos et al. 2001
2610	*M. hominis*	C	U	Pereyre et al. 2002
2611	*Chlamydia trachomatis*	C	T	Misyurina et al. 2004
	E. coli	C	T	Vannuffel et al. 1992
	H. pylori	C	A	Rimbara et al. 2008
	M. hominis	C	T	Pereyre et al. 2002
	M. pneumoniae	C	A, G	Matsuoka et al. 2004; Peuchant et al. 2009; Kawai et al. 2013; Ye et al. 2013
	Neisseria gonorrhoeae	C		Ng et al. 2002
	S. pneumoniae	C	A, G	Tait-Kamradt et al. 2000b; Pihlajamaki et al. 2002; Farrell et al. 2003; Farrell and Felmingham 2004
	S. pneumoniae	C	T	Rantala et al. 2005
	S. pyogenes	C	T	Malbruny et al. 2002

Not all mutations have been shown to solely cause macrolide/ketolide resistance; base substitutions can occur in the background of ribosomal protein changes.

[a]This mutation was not required for clarithromycin resistance in the background of C2611A mutation in (Rimbara et al. 2008).

An analysis of 14 of 217 erythromycin-resistant clinical *S. pneumoniae* isolates collected in Finland in 2002 (Rantala et al. 2005) characterized by polymerase chain reaction (PCR) did not harbor an efflux mechanism, *mef*(E) or *mef*(A), or a target modification mechanism, *erm*(B) or *erm*(A) subclass *erm*(TR), but did have previously identified A2059G mutations in one or more 23S rRNA genes. As had been shown previously, erythromycin minimum inhibitory concentrations (MICs) increased with an increasing number of *rrl* alleles containing A2059G (Tait-Kamradt et al. 2000a), whereas a C2611T mutation was present in all four alleles (Rantala et al. 2005). In *Helicobacter pylori*, only one of the two alleles needs to contain a 23S rRNA mutation to result in macrolide resistance (Hulten et al. 1997). Mutations in *rrl* are also found in combination with mutations in genes encoding L4 or L22 in many species.

Ribosomal Protein Mutations

Mutations in genes encoding ribosomal proteins L4 and L22 in laboratory isolates of *E. coli* and clinical isolates of *S. pneumoniae* can confer erythromycin resistance and reduced telithromycin susceptibility (Tait-Kamradt et al. 2000a; Pihlajamaki et al. 2002). In addition to the changes detailed below, a list of L4 and L22 mutations can be found in Franceschi et al. (2004).

Changes within a highly conserved sequence of *S. pneumoniae* L4 ($_{63}$KPWRQKGTG RAR$_{74}$) can result in decreased susceptibility to macrolides or ketolides (a 500-fold increase to a telithromycin MIC of 3.12 μg/mL for one variation) as well as alter fitness or confer temperature sensitivity of growth (Tait-Kamradt et al. 2000a,b; Farrell et al. 2004). This sequence forms the loop that extends into the PET. Mutations in L4 that have been identified within the conserved sequence encode $_{68}$E$_{69}$, $_{68}$KEG$_{69}$, or $_{68}$GQK$_{69}$ insertions; T$_{94}$I, E$_{30}$K, S$_{20}$N, G$_{71}$R, I$_{78}$V, K$_{68}$S, K$_{68}$Q, $_{69}$VP$_{70}$, $_{69}$TPS$_{71}$, or V$_{88}$I substitutions; $_{69}$GTGR$_{72}$ or $_{64}$P—Q$_{67}$ deletions. In *S. pyogenes* isolates from children treated with azithromycin, amino acid variations in L4 ($_{64}$WR$_{65}$ or $_{69}$TG$_{70}$ deletion; insertion of RA after position 73, $_{73}$RA) were uncovered (Bingen et al. 2002). In *H. influenzae*, L4 amino acid variations (insertion $_{65}$GT, K$_{61}$Q, T$_{64}$K, G$_{65}$D; deletion $_{65}$GR, G$_{53}$A; deletion of $_{66}$RA, A$_{69}$S, T$_{82}$I, D$_{94}$E, D$_{139}$G), some outside the loop region, could provide high-level resistance of up to 128 μg/mL for 14- and 15-membered macrolides (Clark et al. 2002; Peric et al. 2003). *S. aureus* isolates with L4 amino acid changes R$_{168}$S, G$_{69}$A, and T$_{70}$P have been described in CF patients (Prunier et al. 2005). Amino acid variations in L4 from *M. genitalium* (N$_{21}$K, H$_{69}$R, V$_{84}$G, E$_{128}$G, P$_{81}$S, Y$_{135}$P, N$_{172}$S, N$_{172}$S, A$_{114}$V, A$_{116}$V, A$_{114}$S, R$_{45}$K) were found encoded in DNA from the urine of men with nongonococcal urethritis (Shimada et al. 2011), and in the chromosomal DNA isolated from a collection of *M. genitalium* isolates (Jensen et al. 2014). The L4 A$_{209}$T variation was found in chromosomal DNA of *Mycoplasma pneumoniae* isolates from patients (Cao et al. 2010).

Mutations encoding amino acid changes in the carboxy-terminal region of ribosomal protein L22 (e.g., G$_{95}$D, P$_{99}$Q, A$_{93}$E, P$_{91}$S, G$_{83}$E, A$_{101}$P, $_{109}$RTAHIT$_{114}$ tandem duplication) resulted in decreased susceptibility to macrolides and ketolides, although the MICs were not greater than 1 μg/mL in *S. pneumoniae* (Canu et al. 2002; Farrell et al. 2003). A mutation encoding K$_{94}$Q along with a large deletion in the *erm*(B) upstream region was selected by telithromycin in a *S. pneumoniae* isolate with *erm*(B) (Walsh et al. 2003). In *H. influenzae* clinical isolates, MICs increased 4- to 16-fold with insertions or deletions in L22 (G$_{91}$D; insertions of $_{77}$DEGPSM, $_{88}$RAKG, $_{91}$KG, $_{91}$RAG, or $_{91}$RADR; deletions of $_{81}$S, $_{82}$M, $_{91}$KG, $_{95}$R, $_{95}$RI, or $_{96}$ILKR) (Clark et al. 2002; Peric et al. 2003). A deletion of three amino acids in L22 associated with an A2058 mutation has also been reported in a *S. aureus* isolate from CF patients (Prunier et al. 2002). Amino acid changes in L22 from *M. genitalium* (A$_{43}$V, G$_{93}$E + D$_{109}$E, S$_{81}$T, S$_{81}$N, M$_{82}$L, N$_{112}$D, R$_{114}$K, E$_{123}$K) were found in men with nongonococcal urethritis (Ito et al. 2011; Shimada et al. 2011). In *M. pneumoniae*, all 14-membered macrolide-resistant isolates harbored a T$_{508}$C mutation in L22 and, for most, either an

A2058G or A2059G mutation in 23S rRNA (Cao et al. 2010; Jensen et al. 2014).

Resistance to telithromycin in *S. pneumoniae* significantly increases when 23S rRNA methylation/mutations are combined with ribosomal protein mutations. For example, a combination of a truncated leader peptide leading to constitutive synthesis of *erm*(B) conferred a telithromycin MIC of 16 μg/mL (Wolter et al. 2008a), whereas clinical isolates with both a constitutive *erm*(B) and a $_{69}$GTG$_{71}$ to TPS substitution in L4 (Wolter et al. 2007) or a combined A2058T mutation and a three-amino acid deletion in L22 (Faccone et al. 2005), provided high-level telithromycin resistance (≥256 μg/mL). A patient with a *S. pneumoniae* isolate harboring an A2058G mutation in 23S rRNA and an RTAHIT insertion in L22 between amino acid T$_{108}$ and V$_{109}$ resulted in a telithromycin MIC of 16 μg/mL (Perez-Trallero et al. 2003). In addition, a telithromycin-resistant isolate with a MIC of 8 μg/mL was found to contain an *erm*(B) gene, an S$_{20}$N variation in L4, and a number of mutations in 23S rRNA (Reinert et al. 2005). A highly resistant laboratory-generated *S. pneumoniae* strain (MIC, 32 μg/ml) contained a 210-bp deletion in the *erm*(B) upstream region together with a K$_{94}$Q mutation in L22 (Walsh et al. 2003).

erm Genes

A major and widespread mechanism of resistance to the macrolide class of antibiotics is mediated by *erm* genes that encode rRNA methyltransferases that add one or two methyl groups to the exocyclic amino group of A2058 (Figs. 3 and 4) located in the PET of 23S rRNA (Horinouchi and Weisblum 1980; Weisblum 1995a). In addition to conferring resistance to 14-, 15-, and 16-membered macrolides and ketolides, resistance to two other classes of antibiotics, lincosamides and streptogramin B, is imparted, giving the host a MLS$_B$K phenotype (Sutcliffe and Leclercq 2002; Roberts 2008; Schwarz et al. 2016).

As of January 2016, 38 *erm* genes have been reported (see faculty.washington.edu/marilynr). Among the *erm* genes, the most commonly carried is *erm*(B) (36 genera), followed by *erm*(C) (32 genera), *erm*(F) (25 genera), *erm*(X) (15 genera), *erm*(V) (11 genera), *erm*(A) (nine genera), *erm*(G) and *erm*(E) (seven genera each), *erm*(Q) (six genera), *erm*(T) (four genera), *erm*(42) (three genera), *erm*(D) and *erm*(R) (two genera each). The remaining 25 *erm* genes are found in a single genus. Sixteen (46%) of the *erm* genes (*erm*(H), *erm*(I), *erm*(N), *erm*(O), *erm*(R), *erm*(S), *erm*(U), *erm*(W), *erm*(Z), *erm*(30), *erm*(31), *erm*(32), *erm*(34), *erm*(36), *erm*(37), *erm*(38), *erm*(39), *erm*(40), *erm*(41), and *erm*(46)) are unique to environmental bacteria, defined as those species and genera that are primarily found outside of humans and animals.

Inducible or Constitutive MLS$_B$ Phenotype

Depending on the nature of leader sequences upstream of the translational start site, *erm* genes are either inducible by antibiotics or constitutively expressed; examples include *erm*(A) (Murphy 1985), *erm*(B) (Min et al. 2008), *erm*(C) (Gryczan et al. 1980; Horinouchi and Weisblum 1980; Weisblum 1995b), and *erm*(D) (Hue and Bechhofer 1992). For inducible *erm* genes, there are leader sequences upstream of the translational start site that form at least two stem and loop structures, one of which sequesters the ribosomal start site for the resistance gene, and the other upstream stem-loop structure that overlaps ORFs for one (*erm*(C), *erm*(B), *erm*(D)) or two (*erm*(A)) short peptides. Thus, in the absence of an inducing antibiotic, the upstream leader sequence and attending peptide is synthesized, but there is no synthesis of the *erm* gene because of sequestration of its ribosome-binding site. In the macrolide-bound ribosome, a macrolide-stalling motif in the nascent leader peptide is encountered and translation is stalled. The stalled ribosome allows an alternative messenger RNA (mRNA) secondary structure to form, such that the ribosome-binding site for the *erm* gene is exposed and available for translation by a ribosome not bound by erythromycin (Min et al. 2008; Arenz et al. 2014b). The programmed arrest of translation is both inducer (small molecule)- and

leader peptide-specific (Mayford and Weisblum 1990; Vazquez-Laslop et al. 2011; Kannan et al. 2014).

The high degree of variability among the regulatory leader regions of the mRNA transcripts for the different classes of *erm* genes, despite the highly conserved nature of the genes themselves, allows for a variety of phenotypes pertaining to induction by particular antibiotics (Subramaniam et al. 2011). Although most *erm* genes are induced by the 14- or 15-membered macrolides and not by 16-membered macrolides or ketolides, exceptions have been noted. The *erm*(B) subgroup *erm*(AMR) from a clinical strain of *Enterococcus faecalis* (Oh et al. 1998), *erm*(S) subgroup *erm*(SF) (Kelemen et al. 1994), and *erm*(V) subgroup *erm*(SV) (Fujisawa and Weisblum 1981) in 16-membered macrolide-producing *Streptomyces* spp. have been shown to be induced by tylosin and, in the latter, other 16-membered macrolides as well (Kamimiya and Weisblum 1997). Inducible resistance in *Streptomyces* spp. is the most diverse, with induction by lincomycin and streptogramin B in corresponding producers resulting in N^6 dimethylation of 23S rRNA and a MLS_B-resistant phenotype (Fujisawa and Weisblum 1981).

The length of the leader peptide can vary. The Erm(C) leader peptide (ErmCL) is 19 amino acids, with amino acids IFVI (I6–I9) constituting an important macrolide-stalling motif that triggers ribosome pausing. Cryoelectron microscopy (cryo-EM) of the erythromycin-dependent ErmCL-stalled ribosome complex (SRC) (Arenz et al. 2014b) revealed the path of the ErmCL nascent polypeptide chain, its contact with erythromycin, and its interactions with 23S rRNA nucleotides U2506, U2586, and A2062 within the ribosomal tunnel. Interactions of ErmCL amino acids V8 and F7 with U2506 and I6 with U2586 are consistent with experiments that show that mutations in the conserved I6–I9 motif severely reduce ribosome stalling (Vazquez-Laslop et al. 2008; Johansson et al. 2014). An interaction of the amino terminus (minimally I3) of ErmCL with A2062 stabilizes an unusual conformation such that this nucleotide is forced to lie flat against the tunnel wall instead of protruding into the tunnel lumen, thereby allowing an interaction with A2503, consistent with the findings that mutations A2062U/C or A2503G dramatically alleviate ErmCL stalling (Vazquez-Laslop et al. 2008, 2010). In addition, ErmCL was observed to directly interact with the cladinose sugar of erythromycin, providing a structural explanation for how the nascent chain monitors the presence of erythromycin in PET. In the ErmCL-SRC, the PTC is remodeled because of interactions of the ErmCL nascent chain with U2586, U2506, and A2062, promoting a flipped conformation of U2585, which makes it unfavorable for the A-tRNA to fully accommodate, leading to dissociation and translation arrest (Arenz et al. 2014b). Previous crystallographic studies have shown that accommodation of the CCA-end of the A-site tRNA requires movement of nucleotides U2584 and U2585 (Schmeing et al. 2005; Simonovic and Steitz 2009).

Although ketolides lack the C3 cladinose and do not induce *erm*(C) by the mechanism described above, they can promote its expression by inducing ribosomal frame-shifting errors within the *erm*(C) leader ORF (Gupta et al. 2013a). Telithromycin induces a (-1) frameshift within a string of four adenine residues in the last two sense codons of *ermCL*, resulting in a read-through of the stop codon and unmasking of the *erm*(C) start codon because of the subsequent change in secondary mRNA structure. When other macrolides were tested on a model leader construct, it was found that frameshifting was also an intrinsic property (although to different degrees) of 14-membered macrolides as well as other ketolides.

In contrast to ErmCL, the ErmBL is 36 amino acids in length and *erm*(B) is induced by a wider range of 14- and 15-membered macrolides, including those that lack the C3 cladinose or have modifications of this sugar (Arenz et al. 2014a). Cryo-EM of ErmBL-SRC with erythromycin shows that nascent ErmBL travels a unique path in the PET and does not come into contact with the antibiotic, thereby defining a paradigm distinctly different than the one used for *erm*(C) induction (Arenz et al. 2014b). Stalling occurs after 10 amino acids have been polymerized and cryo-EM of the ErmBL-SRC

shows that the P-site and the A-site are filled with the ErmBL-tRNA and the Lys-tRNA (K_{11}), respectively (Min et al. 2008; Vazquez-Laslop et al. 2010). No further peptide bond formation occurs because interactions of amino acids $_9VD_{10}$ and R_7 with U2585 and U2586, respectively, stabilize U2585 in a position that precludes Lys-tRNA from being properly accommodated in the A-site. Notably missing in the ErmBL-SRC was any interaction of the nascent peptide with A2062 that was so critical for ErmCL-mediated ribosome stalling.

Gene regulation by nascent-peptide-dependent ribosome stalling expands beyond antibiotic resistance genes. Other examples in bacteria include translation arrest at the *secM* ORF activating the expression of *secA* (Nakatogawa and Ito 2002; Bhushan et al. 2011) and ribosome stalling of the *tnaC* ORF regulating the expression of the tryptophanase operon (Gong and Yanofsky 2002; Seidelt et al. 2009).

The *erm*(D) subclass *erm*(K) is regulated by both transcription and translation attenuation (Kwak et al. 1991; Choi et al. 1997), whereas *erm*(37) in *Mycobacterium tuberculosis* (Buriankova et al. 2004) has been shown to be activated by the erythromycin-inducible transcription activator WhiB7 (Morris et al. 2005). The *erm*(41) gene intrinsic in *Mycobacterium abscessus* and *M. bolletii* has been shown to be inducible by macrolides and ketolides; however, sequence analysis of the upstream region does not provide compelling evidence for regulation of this gene by either transcription or translation attenuation or by the inducible transcription factor WhiB (Nash et al. 2009).

Constitutive MLS_B resistance can be conferred by a variety of mutations in the leader sequence (Sutcliffe and Leclercq 2002; Subramaniam et al. 2011), and includes deletions of the entire attenuator region for *erm*(C) in clinical isolates of *S. epidermidis* and *S. aureus* (Lampson and Parisi 1986) and for *erm*(B) in *E. faecalis*, *S. agalactiae*, and *S. pneumoniae* (Martin et al. 1987; Rosato et al. 1999; Wolter et al. 2008b) as well as tandem duplications in the attenuator of *erm*(C) of *S. aureus* and *S. equorum* (Oliveira et al. 1993; Lodder et al. 1997), which either destabilize the hairpin structure sequestering the initiation sequences for the methyltransferase or duplicate the initiation sequences, leaving one unsequestered and available for translation. Notably, constitutive *erm*(B)-containing pneumococcal isolates with a higher percentage of 23S rRNA methylation were telithromycin-resistant (Douthwaite et al. 2005; Wolter et al. 2008a).

Clindamycin and 16-membered macrolides with more than an amino sugar at C5 do not induce *erm* expression in most species. This is now understood because both clindamycin and 16-membered macrolides directly interact with the PTC, inhibiting peptide bond synthesis; thus, is it unlikely that the synthesis of nascent peptide longer than a few amino acids could be synthesized, too short for the ribosome to sense or the nascent peptide to interact with the antibiotic (Tenson et al. 2003). Clinical isolates of *S. pyogenes* or *S. agalactiae* with variations in the leader sequence, including point mutations, insertions, deletions, and duplications, were shown to be resistant to both erythromycin and clindamycin, showing that constitutive resistance yields an MLS_B phenotype (Culebras et al. 2005; Doktor and Shortridge 2005). In this study, three isolates with a 44-base duplication/insertion corresponding to bases 188 to 231, duplicating the *erm*(A) ribosomal binding site and start site, and one isolate with a 68-bp deletion of the entire leader peptide 2 region, were also resistant to the ketolide telithromycin.

Why are most *erm* genes inducible rather than constitutively expressed? Ribosome methylation at A2058 exerts a fitness cost because of the change in the ribosome's ability to sense/respond to nascent peptides, thereby changing the expression of a number of cellular polypeptides (Ramu et al. 2009; Gupta et al. 2013b; Wilson 2014). Thus, deregulation of translation may well explain why bacteria prefer to retain the ability to become conditionally resistant.

cis-Acting Peptides

Translation of a pentapeptide encoded in *E. coli* 23S rRNA can cause macrolides to dissociate from the ribosome, thereby conferring macrolide resistance (Tenson et al. 1996). Other *cis*-

acting peptides (resistance conferred only to a ribosome on which the peptide is synthesized) have been identified using a random-library approach, providing a consensus sequence, fMet-(bulky/hydrophobic)-(Leu/Ile)-(hydrophobic)-Val for erythromycin resistance; other consensus peptides specific for different macrolides (e.g., oleandomycin, ketolides, 15-membered macrolides) were also identified, consistent with the ability of the PET to distinguish small changes in antibiotic/nascent peptide interactions (Tenson et al. 1997; Vimberg et al. 2004). When key amino acids are synthesized in specific short peptides, the affinity of the macrolide/ketolide for its binding site is weakened, but removal of the antibiotic from the ribosome is most likely when the pentapeptide is removed from the peptidyl-tRNA by class I release factor (Lovmar et al. 2006).

Rlm Methyltransferases

The importance of modifications to 23S rRNA is not completely understood. However, for tylosin producer *Streptomyces fradiae*, monomethylation of G748 and A2058 by rRNA methyltransferase $RlmA^{II}$ and Erm(N), respectively, is needed for self-preservation (Liu and Douthwaite 2002b; Takaya et al. 2013). Certain Gram-positive bacteria like *S. pneumoniae* have an intrinsic chromosomal *rlmA*^{II} gene (Liu and Douthwaite 2002a). Molecular modeling shows that the methyl group of G748 stabilizes the binding of telithromycin to the ribosome by moving the alkyl–aryl arm of telithromycin toward the aromatic rings of A752 in helix 35 (Fig. 3 shows the positions of A752 and A2058) (Takaya et al. 2013). Mutations in *rlmA*^{II} preventing methylation of G748 in *S. pneumoniae* isolates that also harbor a constitutive *erm*(B) result in 16- to 32-fold greater resistance to telithromycin (Takaya et al. 2013). Just to keep things interesting, *S. pneumoniae* has another methyltransferase, RlmCD, which mediates a methyl transfer to both U747 (in helix 35) and U1939. Recent data have shown that $RlmA^{II}$ prefers U747-methylated 23S rRNA as a substrate; thus, when these two methyltransferases work sequentially, the binding of telithromycin to the ribosome is facilitated and, in their absence, telithromycin resistance occurs when there is dimethylation of A2058 by an *erm* methylase (Shoji et al. 2015).

Acquired Macrolide Efflux Mechanisms

Mef Family. Mef pumps are members of the major facilitator superfamily and have 12-transmembrane domains connected by hydrophilic loops, with both the amino and carboxyl termini located in the cytoplasm (Paulsen et al. 1996; Pao et al. 1998). They are antiporters with a binding site for macrolide antibiotics and have a rocker-switch type of movement, in which conformational changes in the protein are elicited to efflux a macrolide in exchange for a proton (Law et al. 2008). The *mef* genes are largely found among Gram-positive bacteria, but have also been reported in Gram-negative species (see faculty.washington.edu/marilynr; Ojo et al. 2004). The two major subclasses, *mef*(A) (Clancy et al. 1996), first identified in *S. pyogenes* isolates, and *mef*(E) (Tait-Kamradt et al. 1997), initially identified in *S. pneumoniae*, were characterized as providing resistance to 14- and 15-membered macrolides but not 16-membered macrolides, lincosamides, or streptogramin B, thereby affording the M (macrolide-resistant only) phenotype (distinguishable from the MLS_B phenotype). Both pumps are collectively categorized as *mef*(A) because of their >80% amino acid sequence identity (Roberts et al. 1999). However, the two genes are carried on distinct genetic elements; Tn*1207.1*, Tn*1207.3*, φ10394.4, or φm46.1 for *mef*(A) (Santagati et al. 2000, 2003; Giovanetti et al. 2003; Varaldo et al. 2009) and a macrolide efflux genetic assembly (megacomplex and derivative transposons Tn*2009* and Tn*2010* for *mef*(E)) (Gay and Stephens 2001; Del Grosso et al. 2002, 2004, 2006; Varaldo et al. 2009). Interestingly, although the megacomplex does not carry a transposase or recombinase as in Tn*1207.1*, the related *mef*-family complexes carry an adjacent ATP-binding cassette (ABC)-type transporter gene with sequences encoding two fused nucleotide-binding domains but no membrane-spanning domains, known as *mel* or *msr*(D) (because of its similarity to the *S. aureus*

msr(A) gene) (Gay and Stephens 2001; Del Grosso et al. 2002). The *msr*(D) gene is adjacent to and cotranscribed with *mef*(E) in the presence of inducers like erythromycin. The exact function of Msr(D) in streptococci has yet to be fully elucidated, but coexpression of *msr*(D) and *mef*(E) is required for high-level macrolide efflux in *S. pneumoniae*, and both proteins interact synergistically to increase macrolide resistance in *E. coli* (Ambrose et al. 2005; Nunez-Samudio and Chesneau 2013). In *E. coli*, a physical association of Msr(D) and Mef(E) was shown using a mef(E)-green fluorescent protein (GFP) fusion, in which it appeared that Msr(D) directed Mef(E)-GFP to the cell poles, possibly assisting in the assembly of Mef(E) in the membrane and/or enhancing macrolide efflux as part of a composite transporter (Nunez-Samudio and Chesneau 2013).

Owing, in part, to the genomic plasticity and natural competency of *S. pneumoniae*, mega has been found in multiple chromosomal locations as well as inserted into other composite mobile elements carrying genetic markers of multiple *Streptococcus* species (Chancey et al. 2015a). Conjugal transfer rates of Tn*1703.1* carrying *mef*(A) have been found to be highly variable between different *S. pyogenes emm*-types, ranging in frequencies of 1.13×10^{-6} to 7.2×10^{-8} in various isolates, with higher frequencies in *emm1* and *emm4* isolates (Hadjirin et al. 2013). The *mef*(A) gene has also been found on a large chimeric chromosomal element that also carries the *tet*(O) gene, an element that lends a distinct *Sma*I-typable pulse-field gel electrophoresis profile to isolates carrying the construct (Brenciani et al. 2004; Bacciaglia et al. 2007).

A novel *mef*(B) gene found in *E. coli* porcine isolates located near *sul3* on plasmids has been described with 38% protein identity (62% similarity) to Mef(A) (Liu et al. 2009a). When *mef*(B) was cloned into a plasmid and transformed into *E. coli* JM109, transformants had the M phenotype. The plasmid location as well as the genetic organization of the *mef*(B) gene were distinct from its organization in conjugative transposons. The GC content (44.95%) was lower than that of *E. coli*, suggesting horizontal transition from another organism.

More recently, a novel subclass *mef*(C) gene was identified, along with a macrolide phosphotransferase *mph*(G), on plasmid pAQU1 isolated from marine bacteria including *Vibrio* and *Photobacterium* (Nonaka et al. 2015). Another subclass, termed *mef*(I) and first identified in isolates of *S. pseudopneumoniae* (Cochetti et al. 2005), has since been isolated from *S. pneumoniae* located on a novel IQ element inserted into defective Tn*5252* and Tn*916* sequences along with a unique *msr*(D) gene variant and *catQ*, a chloramphenicol acetyltransferase (Mingoia et al. 2007). The *mef*(O) subclass, which has a high degree of similarity to *mef* genes from *S. dysgalactiae*, was identified in *S. pyogenes* isolates from Norway (Sangvik 2005; Blackman Northwood et al. 2009). In addition to these, *mef*(B) and *mef*(G) genes were identified in *S. agalactiae* and group G streptococci, respectively, conferring an M phenotype and showing high degrees of sequence identity to each other, although <90% sequence identity to *mef*(A) or *mef*(E) (Amezaga and McKenzie 2006; Cai et al. 2007). However, *mef*(C), *mef*(G), *mef*(I), and *mef*(O) have >80% amino acid sequence homology with class *mef*(A), so none are recognized as a separate class at the website maintained by Marilyn Roberts (see faculty.washington.edu/marilynr; Roberts et al. 1999).

Derivatives of *S. pyogenes* sequence type 39 have been found carrying multiple mosaic *mef* gene variants encompassing the 5′ and terminal portions of *mef*(A) combined with a region of *mef*(E) spanning the majority of bases 570–1100 (*mef*(A) sequence numbering used), often on a φm46.1-like element and in conjunction with the tetracycline resistance gene *tet*(O) (Blackman Northwood et al. 2009; Del Grosso et al. 2011). Some composite resistance genes containing *mef*-family sequences have been detected in which the *msr*(D) gene was not readily amplified by PCR; however, it was not clear whether this was because of sequence variance or absence of the genes (Cerda Zolezzi et al. 2004).

Induction of the *mef*(E)-*msr*(D) operon has been linked to the presence of substrate macrolides (Daly et al. 2004; Ambrose et al. 2005;

Wierzbowski et al. 2005), with induction occurring by most 14- and 15-membered macrolides, including ketolides (Chancey et al. 2011). The expression of the efflux operon in response to drug exposure appears to be correlated with the presence of a free hydroxyl at the 2′ position of a monosaccharide amino sugar like desosamine at position C5, rather than correlated to macrocyclic ring size or C3 sugar composition (see Fig. 1 for macrolide structures). For example, troleandomycin with an acetate substitution at the 2′ hydroxyl of desosamine, does not induce *mef*(E)-*msr*(D) (Chancey et al. 2011) nor do the majority of 16-membered macrolides with a disaccharide at C5 (see Fig. 2 for 16-membered macrolide structures). *mef*(E)-*msr*(D)-inducing 16-membered macrolides, such as tilmicosin and rosamicin, and the 14-membered ketolide telithromycin have C5 monosaccharides that bind in the ribosome in locations similar to efflux substrates erythromycin and azithromycin, but distinct from weakly/non-inducing macrolides with C5 disaccharides or modifications to C3 or C5 sugars (Chancey et al. 2011). Although both rosamicin and tilmicosin induced expression of *mef*(E)/*msr*(D), only tilmicosin appears to be a substrate for the Mef(E)/Msr(D) pumps (Chancey et al. 2011).

mef genes are regulated by transcription attenuation, with the induction of the *mef*(E)/*msr*(D) operon occurring by anti-attenuation of transcription in the presence of inducing macrolides; however, there is also evidence that other regulatory mechanisms influence the control of *mef*(E)/*msr*(D). There is a leader peptide encoded 34 bp upstream of the *mef*(E) start codon that is required for full expression of *mef*(E)/*msr*(D) (Subramaniam et al. 2011; Chancey et al. 2015b). Macrolide-bound ribosomes stall in the leader peptide, causing a shift in mRNA conformation, similar to induction of activity of *erm* methyltransferase genes (Chancey et al. 2015a). The *mef*(E) gene is also induced by unrelated structures such as LL-37, a cationic antimicrobial peptide produced in human macrophages, and two related murine homologs (Zahner et al. 2010). LL-37 may induce *mef*(E) by a different mechanism, but induction by either LL-37 or erythromycin confers resistance to both.

Msr Family. There are four macrolide efflux *msr* types, with each class having $\leq$80% amino acid homology with any member of any other type (see faculty.washington.edu/marilynr). All Msr classes have ATP-binding motif sequence homology with the ATP-binding transport superfamily (Ross et al. 1990). *msr*(A) or *msr*(B) genes (encoding a polypeptide homologous to the carboxyl terminus of Msr(A)) were first identified in *S. epidermidis* and *S. xylosus*, respectively (Ross et al. 1990; Milton et al. 1992), but are now characterized as a single class. These genes have also been described in clinical *S. aureus* isolates (Wondrack et al. 1996; Matsuoka et al. 1999, 2003). The *msr*(A) family genes confer resistance to 14- and 15-membered macrolides and streptogramin B (MS phenotype), and low-level resistance to ketolides (Ross et al. 1995, 1996; Wondrack et al. 1996; Canton et al. 2005; Reynolds and Cove 2005; Vimberg et al. 2015). Related *msr*-family efflux genes have been isolated from other genera, including *Enterococcus* (*msr*(C), *msr*(A)), *Streptococcus* (*msr*(D), also known as *mel*), *Pseudomonas* (*msr*(A)), *Corynebacterium* (*msr*(A)), and in various environmental isolates (*msr*(E)) (Portillo et al. 2000; Ojo et al. 2006; Varaldo et al. 2009; Desmolaize et al. 2011; Roberts 2011).

The structure of the Msr(A) protein is classified as a class 2 ABC-transporter, containing two ATP-binding domains and a long Q-linker region, but not a typical membrane-spanning region. Thus, there exists more than one hypothesized mechanism for Msr(A) function (Ross et al. 1995, 1996; Reynolds et al. 2003). Theories describing the mechanism of action for Msr(A)-linked resistance involve interaction of Msr(A) with the ribosome, blocking binding to the 23S rRNA target site that overlaps macrolides and streptogramin B, or an ATP-dependent efflux pump activity mediated by interaction with membrane-spanning binding proteins (possibly Mef(E)) (Kerr et al. 2005; Nunez-Samudio and Chesneau 2013), or as a structure that helps with the localization and/or assembly of Mef(E) into the membrane (Nunez-Samudio and Chesneau 2013). Studies

with efflux pump inhibitors show Msr(A) function is uninhibited by reserpine, a common Gram-positive efflux pump inhibitor, but efflux activity was inhibited by arsenate, dinitrophenol, or CCCP, supporting the ATP-dependent function of the pump. To date, there has been no direct evidence to support the hypothesis of ribosome protection and recent evidence suggests that Mef(E) and Msr(D) may form a composite efflux pump (Nunez-Samudio and Chesneau 2013).

A recent study that examined the nature of telithromycin resistance in mutants selected in *S. aureus* RN4220 recombinantly expressing *msr*(A) found that mutations mapped to *clpX*, a protein that functions as both the substrate-recognizing component of the ClpXP proteolytic system and as a ClpP-independent chaperone for protein–DNA and protein–protein complexes (Burton et al. 2001). The decreased susceptibility of telithromycin (and erythromycin) was Msr(A)-mediated and related to loss-of-function mutations in ClpX only (Vimberg et al. 2015).

The *msr*(A) gene was initially isolated on a *S. epidermidis* plasmid designated pUL5050, along with a single-domain ATP-binding protein (*stpA*) and a hydrophobic protein (*smpA*), which are similar to *S. aureus* chromosomal genes *stpC* and *smpC*, but it was shown that these genes played no role in conferring macrolide resistance (Ross et al. 1996). A variety of hybrid resistance plasmids have been found carrying *msr*(A) and similar genes along with other resistance elements; pMS97 carrying *msr*(A) and the macrolide-inactivating phosphotransferase *mph*(C) (Matsuoka et al. 2003) and hybrid plasmids mediating combinations of penicillinase, tetracycline-efflux, and ribosomal methylation functions (Argudin et al. 2014) as examples. Expression of *msr*(A) is mediated in a similar manner to *erm* genes, via translation attenuation mechanisms, but requires higher amounts of inducer (Ross et al. 1990, 1996; Subramaniam et al. 2011). If the 320-bp control region upstream of *msr*(A) is deleted, the strain is constitutively macrolide-streptogramin B–resistant, analogous to deletions or mutations that destroy the secondary structure that is attenuated by drug-dependent stalling within the leader peptide of *erm* genes.

MACROLIDE INACTIVATION

Macrolide Esterases

Inactivation of erythromycin by hydrolysis was first shown to be widespread in Enterobacteriaceae isolated from the human fecal flora and was usually associated with erythromycin therapy (Barthelemy et al. 1984; Andremont et al. 1985, 1986; Arthur and Courvalin 1986). Hydrolytic inactivation of macrolides by esterases specifically involves 14- and 15-membered macrolides; josamycin, midecamycin, rosaramycin, and spiramycin are not substrates (Arthur and Courvalin 1986; Arthur et al. 1987; Morar et al. 2012).

Two plasmid-encoded esterases, *ere*(A) and *ere*(B), conferring high-level erythromycin resistance (MIC $\geq$ 1 mg/mL), have been isolated from *E. coli*. The *ere*(A) gene on the self-transmissible plasmid pIP1100 encodes a product with a molecular weight of 37,765. The *ere*(B) gene, encoding an enzyme with a molecular weight of 51,000, was first identified on the self-transmissible plasmid pIP1527, which also contained the *erm*(B) gene, formerly known as *erxA* and *ermAM*, encoding an rRNA-methylating enzyme commonly found in streptococci (Arthur and Courvalin 1986). Based on GC content and codon usage, *ere*(A) (GC content 50%) is thought to have originated in Gram-negative bacteria, whereas *ere*(B) (GC content 36%), although originally discovered in *E. coli*, is thought to have originated from Gram-positive bacteria (Arthur et al. 1987). The gene for *erm*(B) is linked to *ere*(B) on plasmid pIP1527, and their physical linkage may be responsible for codissemination of the genes (Arthur et al. 1987). Similar codon usage in *ere*(B) and *erm*(B) suggests a similar Gram-positive bacterial origin; however, the separation of both genes on pIP1527 by GC-rich sequences suggests that both genes were integrated into plasmid pIP1527 by separate genetic events (Arthur and Courvalin 1986). It has been shown that coexpression of *ere*(B) and *erm*(B) more than

additively contributes to erythromycin resistance in *E. coli* (Arthur and Courvalin 1986).

Ere(A), a type I esterase, and Ere(B), a type II esterase, both hydrolyze the lactone ring in 14-membered macrolides (Morar et al. 2012); however, the two enzymes are only weakly related with 25% protein sequence identity. Using a genomic enzymology approach, the catalytic mechanisms of the "erythromycin esterase superfamily" enzymes were compared (Morar et al. 2012). Ere(A), Ere(B), and two related enzymes from *Bacillus cereus*, Bcr135 and Bcr136, whose three-dimensional structures had previously been determined, were studied. Ere(A), Ere(B), and Bcr136 were found to be distinct, with only Ere(A) inhibited by chelating agents and hypothesized to contain a noncatalytic metal. Data from kinetic, mutagenesis, and modeling studies are consistent with all of the erythromycin esterases sharing a common catalytic mechanism, and efforts to detect a tightly bound metal in Ere(B) and Bcr136 were unsuccessful, leaving the hypothesis that Ere enzymes do not require a metal ion for their catalytic mechanism. Thus, the metal dependence of Ere(A) may be structural. A histidine residue, H_{46} (Ere(B) numbering), was found to be essential for catalytic function and proposed to serve as a general base in the activation of a nucleophilic water molecule. Ere(A) and Ere(B) substrate profiles differed. Ere(B) inactivated erythromycin, clarithromycin, roxithromycin, and azithromycin, but was inactive against the ketolide telithromycin. Ere(A) was unable to inactivate either telithromycin or azithromycin.

In recent years, the *ere*(A2) gene, a variant of *ere*(A) located in a class 1 integron cassette, has been found in *Enterobacter aerogenes*, *E. cloacae*, *E. coli*, *Klebsiella oxytoca*, *K. pneumoniae*, *Providencia stuartii*, *Pseudomonas* spp., *Salmonella enterica*, and *Vibrio cholera* (Chang et al. 2000; Peters et al. 2001; Kim et al. 2002; Thungapathra et al. 2002; Plante et al. 2003; Verdet et al. 2006; Abbassi et al. 2008; Chen et al. 2009; Krauland et al. 2010). Although macrolide antibiotics are generally not used in the treatment of nongastrointestinal infections caused by enteric bacteria, the spread of *ere*(A2) in Enterobacteriaceae is concerning because macrolides are often used in the treatment of traveler's diarrhea, and erythromycin is a common treatment of cholera in children and pregnant women (see cdc.gov/cholera/doc/recommend-anitbiotics-treatment.docx).

Phosphotransferases

Macrolide phosphotransferases are macrolide-inactivating enzymes widespread in Gram-negative and Gram-positive bacteria (Sutcliffe and Leclercq 2002; Roberts 2008) that, by in silico analysis, are in the same family as aminoglycoside and protein kinases (Shakya and Wright 2010). The first reported purifications of macrolide-2′-phosphotransferases were from macrolide-resistant *E. coli*, and this mechanism was soon shown to be prevalent in *E. coli* clinical isolates in Japan (O'Hara et al. 1989; Kono et al. 1992; Taniguchi et al. 2004). Macrolide 2′-phosphotransferases, commonly found on mobile genetic elements, are inducible (e.g., *mph*(A)) or constitutively expressed (e.g., *mph*(B)) intracellular enzymes capable of transferring the γ-phosphate of nucleotide triphosphate to the 2′-OH group of 14-, 15-, and 16-membered-ring macrolide antibiotics, thereby disrupting the macrolide's key interaction with A2058. Although early studies showed Mph enzymes could use ATP, more recent work with Mph(A) has shown a preference for GTP under physiologically relevant in vitro assay conditions (Shakya and Wright 2010). Expression of *mph*(A) is induced by erythromycin, and, recently, the structure of the MphR(A) repressor protein, a negative regulator of *mph*(A) expression, has been solved uncomplexed and complexed with erythromycin to 2.00 Å and 1.76 Å resolutions, respectively (Zheng et al. 2009). Erythromycin binds with a stoichiometry of 1:1 to each monomer of the functional MphR(A) dimer in a large hydrophobic cavern composed of residues from α helices of one monomer and the dimeric interface of the other monomer that appears to close around the ligand as it binds (Zheng et al. 2009).

Seven distinct macrolide phosphotransferases have been identified to date (see faculty.washington.edu/marilynr). The first identified

phosphotransferases, Mph(A) and Mph(B), share 37% amino acid identity (O'Hara et al. 1989; Kono et al. 1992). The G+C contents of the *mph*(A) gene (66%) (Noguchi et al. 1995) and the *mph*(B) gene (38%) (Noguchi et al. 1996) differ significantly from each other and the G+C content of *E. coli* chromosome (50%) (Muto and Osawa 1987), suggesting an exogenous nature of their origins. The *mph*(B) has been functionally expressed in both *E. coli* and *S. aureus*, providing a first indication of the potential promiscuity of this macrolide resistance mechanism (Noguchi et al. 1998). In contrast, the *mph*(A) gene could be expressed in *E. coli* but not in *S. aureus*, presumably because of its relatively higher G+C content relative to that of the *S. aureus* chromosome (33%) (Muto and Osawa 1987). The *mph*(C) gene, formerly *mphBM*, along with genes encoding the Msr(A) efflux pump, and Erm methyltransferase, were first identified as naturally occurring on a transmissible plasmid in *S. aureus* clinical isolate (Matsuoka et al. 1998). The sequence of Mph(C) showed 67% amino acid similarity to Mph(B) from *E. coli.* In this study, expression of *mph*(C) in *S. aureus* was shown to be highly dependent on the presence of a portion of the gene encoding the Msr(A) efflux pump; however, the nature of this dependence is not fully understood (Matsuoka et al. 2003).

Of the two enzymes originally found in *E. coli,* Mph(A) preferentially phosphorylates 14- and 15-membered ring versus 16-membered macrolides, whereas Mph(B) phosphorylates 14- and 16-membered macrolides efficiently (Kono et al. 1992; O'Hara and Yamamoto 1996). Clear substrate specificity of these enzymes was shown by recombinant overexpression of *mph*(A), *mph*(B), and *mph*(C) in an isogenic strain background using an efflux-deficient laboratory *E. coli* strain (Chesneau et al. 2007). This study found that Mph(A) conferred resistance to erythromycin, telithromycin, azithromycin, and spiramycin. The closely related Mph(B) and Mph(C) enzymes both conferred resistance to erythromycin, spiramycin, and telithromycin, but no activity against azithromycin was observed. Further, functional expression of *mph*(C) in *E. coli* showed phosphotransferase activity in the absence of *msr*(A) (Chesneau et al. 2007); however, the same plasmid did not confer macrolide resistance in *S. aureus* for reasons unknown, similar to previous observations (Matsuoka et al. 2003).

Residues shown to be important for enzymatic activity were found in the same relative positions in an alignment of Mph(A), Mph(B), and Mph(C), making it difficult to attribute substrate specificities to specific sequence variations (Chesneau et al. 2007). Site-directed mutagenesis of five aspartic acid residues (D_{200}, D_{209}, D_{219}, D_{227}, and D_{231}) thought to be located in the active site of Mph(B) based on alignments with the aminoglycoside phosphotransferase APH(3′)-IIa (Wright and Thompson 1999), showed that replacements of all aspartic acid residues with alanine, except for D_{227}, completely inactivated Mph(B). The D_{227}A mutant retained 7% of the wild-type activity and showed altered substrate specificity with regard to 16-membered ring macrolides, suggesting a role for D_{227} in substrate recognition (Taniguchi et al. 1999). A similar site-directed mutagenesis study investigated conserved histidines H_{198} and H_{205} located in the active site of Mph(B) (Taniguchi et al. 2004). In this study, an H_{198}A mutant retained 50% of the specific enzymatic activity, suggesting that H_{198} was not a catalytically essential residue. In contrast, the H_{205}A mutant retained only 0.7% of wild-type levels of activity, and an H_{205}N mutant retained greater than half of wild-type levels, suggesting that H_{205} was essential for catalysis. Based on alignments with the active site in the structure of an aminoglycoside phosphotransferase, H_{205} was proposed to contact the γ-phosphate of ATP through magnesium and aid in the transfer of phosphate from ATP to the 2′-hydroxyl of the desosamine.

Macrolide phosphotransferases are widespread in bacteria of clinical, veterinary, agricultural, and environmental origins. Genes encoding Mph enzymes are usually found on mobile genetic elements containing other macrolide resistance genes and genes conferring resistance to other antibiotic classes. The *mph*(A) gene has been found on plasmids that encode CTX-M extended-spectrum β-lactamases originating

in *E. coli* ST131 (Woodford et al. 2009; Sandegren et al. 2012) and 16S rRNA methyltransferases (i.e., *armA*) that encode aminoglycoside resistance. The *mph*(A) gene has also been detected in multidrug-resistant (MDR) and KPC carbapenemase-producing *K. pneumoniae* (Soge et al. 2006; Sandegren et al. 2012; Lee et al. 2014), *Shigella* spp. isolates (Boumghar-Bourtchai et al. 2008; Howie et al. 2010; Gaudreau et al. 2014), in globally collected MDR and susceptible *E. coli* isolates (Phuc Nguyen et al. 2009), as well as other Gram-negative pathogens.

The *mph*(C) gene appears to be widespread in staphylococci and has been found in isolates from horse skin (Schnellmann et al. 2006), bovine mastitis (Luthje 2006; Li et al. 2015), and dogs, cats, and pigs (Luthje and Schwarz 2007). *mph*(C) has also been identified in corynebacteria from healthy human skin (Szemraj et al. 2014) and, interestingly, in *Stenotrophomonas maltophilia* (Alonso et al. 2000).

A partial sequence of *mph*(D) (AB048591) has been described from *Pseudomonas aeruginosa* clinical isolate M398 from Japan (Nakamura et al. 2000). Inactivation of oleandomycin was dependent on either ATP or GTP addition to crude extracts and the inactivated product chromatographed with the standard oleadomycin 2′-phosphate. Although the strain was resistant to 14-, 15-, and 16-membered macrolides, crude extracts only inactivated 14-membered macrolides, with some activity (15% inactivation) toward azithromycin. Because this PCR product had only 53% identity with other *mph* genes, it was given a separate designation. Variants of this gene have also been described in *E. coli*, *Klebsiella*, *Pantoeae*, *Proteus*, and *Stenotrophomonas* (see faculty.washington.edu/marilynr).

Macrolide phosphotransferase genes designated as *mph*(E) have been found in the chromosomes of *Acinetobacter baumannii* (Poirel et al. 2008) and bovine respiratory *Pasturella multocida* and *Mannheimia haemolytica* isolates (Desmolaize et al. 2011; Kadlec et al. 2011). They are also transferable on plasmids, including mobile, broad-host range IncP-1β plasmids, and have been described in *Serratia marscescens* (Bae et al. 2009), *K. pneumoniae* (Shen et al. 2009; Jiang et al. 2010), *A. baumannii* (Poirel et al. 2008; Zarrilli et al. 2008), *E. coli* (GenBank #FJ187822, partial sequence) (Gonzalez-Zorn et al. 2005; Bercot et al. 2008), *Citrobacter freundii* (Golebiewski et al. 2007), and in plasmid DNA from uncultured bacterium from wastewater treatment facilities (Schluter et al. 2007; Szczepanowski et al. 2007) (note that although the investigators designate the *mph* gene as *mph*(E), it is listed at macrolide nomenclature center as *mph*(F)). *mph*(A) and *mph*(E) genes are often found in the context of a macrolide resistance operon, either *mph*(A)-*mrx*-*mphR*(A) or *mphR*(E)-*mph*(E)-*mrx*(E), and the operons are bordered by inverted repeat motifs of IS elements, suggesting that the latter could play an important role in the acquisition and spread of these resistance genes (Noguchi et al. 1995; Poole et al. 2006; Szczepanowski et al. 2007). The deduced gene product of *mphR* is a transcriptional regulator (where studied, a negative regulator of *mph*(A) gene expression; Noguchi et al. 2000) of the TetR/AcrR family, whereas the *mrx* genes encode a putative transmembrane transport protein; both are needed for high-level expression of macrolide resistance.

The most recently identified macrolide phosphotransferase, *mph*(G), has been found in *Vibrio* spp. and photobacteria in the seawater of fish farms (Nonaka et al. 2015).

SURVEILLANCE OF MACROLIDE RESISTANCE AND CHARACTERIZATION OF MOLECULAR MECHANISMS

For treatment of community-acquired pneumonia, a 14- or 15-membered macrolide plus a β-lactam is part of the regimen for patients with risk factors and is recommended as a single agent in patients without risk factors (Mandell et al. 2007). In a study that assessed the macrolide failures in patients with pneumococcal bacteremia, *mef*(A) and *erm*(B) were equally overrepresented, but MIC increases >1 μg/mL were not associated with any greater failure rate (Daneman et al. 2006), thus showing that the lower level resistance generally seen in pneumococci harboring *mef*(A) is clinically signif-

icant. Mutations in 23S rRNA are more frequently found where there are chronic or prolonged treatment regimens, such as for CF patients and those with *M. pneumoniae* or *H. pylori* infections (Table 2). The studies can be difficult to compare as investigators choose different genes to monitor their surveillance population.

Surveillance studies (published largely in 2006–2015) find macrolide resistance rates ranging from <10% (Columbia, Hidalgo et al. 2011; Alaska, Rudolph et al. 2013) to >60% (Asia, Song et al. 2004; Lebanon, Taha et al. 2012) in pneumococci. For group A streptococci, there was also a wide range in macrolide-resistant rates, varying from 2% (Utah, Rowe et al. 2009; Scotland, Amezaga and McKenzie 2006; The Netherlands, Buter et al. 2010) to 98% in *S. pyogenes* (Chengdu, China, Zhou et al. 2014). It has been shown that macrolide resistance rates can increase with erythromycin usage (Seppala et al. 1997) and intermediate/long-acting macrolide consumption (Italy, Cornaglia et al. 1996; Spain, Perez-Trallero et al. 1998; Slovenia, Cizman et al. 2001), but not all countries have increasing macrolide resistance paralleling increase in macrolide consumption (Portugal, Silva-Costa et al. 2015). Clonality can also play a role as was seen in erythromycin-resistant *S. pyogenes* in Pittsburgh, where all of the macrolide resistance (48%) was the result of a single strain of *S. pyogenes* (Martin et al. 2002), showing resistance rates in one city or small region are possibly not representative for an entire country. Other factors yet determined also play a role. The rates of macrolide resistance in group B streptococci, including *S. agalactiae*, range from 4% to 5% (Scotland, Amezaga and McKenzie 2006; The Netherlands, Buter et al. 2010) to 40% (France, Bergal et al. 2015; Tunisia, Hraoui et al. 2012) and viridans streptococci generally have higher rates, ranging from 27% (Turkey, Ergin et al. 2006) to 63% (Canada, Thornton et al. 2015). Thus, it is important to continue surveillance and monitor resistance rates locally and globally.

A high rate of azithromycin-resistant streptococci was resident and characterized in adults with CF, with half of the isolates harboring A2058G or A2059G mutations in 23S rRNA (Thornton et al. 2015). In *S. aureus* isolates from adult and children patients with CF (Tkadlec et al. 2015), 52% of the macrolide-resistant isolates had 23S rRNA or L4 ribosomal mutations. Mechanisms of macrolide resistance appear to be different in Gram-negative isolates from children with CF (Roberts et al. 2011). In patients participating in a randomized placebo-controlled trial with azithromycin, there was 25.5% frank macrolide resistance in *H. influenzae* with all but one of the remaining isolates intermediate to azithromycin. Rather than ribosomal protein mutations, *erm*(B) and *erm*(F) were frequently identified, usually in combination with *mef*(A); 23S rRNA mutations were not interrogated.

The rates of macrolide resistance in methicillin-resistant *S. aureus* (MRSA) and coagulase-negative staphylococci remain high (44%–100%), with *erm* genes as the most predominant mechanisms. Macrolide resistance in MRSA is significantly higher than in methicillin-susceptible *S. aureus* (MSSA) and, in a recent study in Turkey, there was a sevenfold difference in macrolide resistance between MRSA and MSSA (Gul et al. 2008; Yildiz et al. 2014; Aydeniz Ozansoy et al. 2015). The rates of macrolide resistance in coagulase-negative staphylococci vary from 44% (*Staphylococcus saprophyticus*, France, Le Bouter et al. 2011) to 100% (*S. haemolyticus*, Poland, Brzychczy-Wloch et al. 2013; *S. hominis*, Poland, Szczuka et al. 2015) in surveillance studies (Table 2). Msr-mediated efflux appears to be increasing in staphylococci, often in conjunction with an *erm* gene. A study of isolates collected from European hospitals in 1997–1998 found *msr* genes in only 13% of MSSA isolates and did not detect the gene in MRSA (Schmitz et al. 2000). Similarly, a surveillance of isolates from French hospitals published in 1999 showed only 2.1% of MRSA/MSSA isolates carrying *msr*(A) resistance genes (Lina et al. 1999). More recent studies have shown *msr* genes present at rates ranging from 1.6% (Iran, Shahsavan et al. 2012) to 79% (Spain, Argudin et al. 2014; Aydeniz Ozansoy et al. 2015) of *S. aureus* and 15% (Tunisia, Bou-

Table 2. Surveillance and mechanisms of macrolide resistance in clinical isolates

Species	Percent macrolide resistance	Number of isolates	Macrolide-resistant mechanism						Country or region	Isolation year(s)	References
			erm[a]	*mef*(A)	*erm*[a] + *mef*(A)	Target[b]	*msr*(A)	*mph*(A)[c]			
Streptococcus pneumoniae	36.5%	20,142	16.3%	9.8%	3.6%	0.4%	ND	ND	Global	2001–2005	Felmingham et al. 2007
S. pneumoniae	9%–14%	15,982	57%	27%	15%	1%	ND	ND	South Africa	2000–2005	Wolter et al. 2008a
S. pneumoniae	8%–22%	12,759	36.9%	51.0%	8.0%	ND	ND	ND	Canada	1998–2008	Wierzbowski et al. 2014
S. pneumoniae	37.2%	7083	55%	30.6%	12.0%	ND	ND	ND	Global	2003–2004	Farrell et al. 2008
S. pneumoniae	35.3%	6747	18.8%	53.8%	24.1%	1.7%	ND	ND	United States	2005–2006	Jenkins and Farrell 2009
S. pneumoniae	9.2%	2923	12.0%	77.0%	7.0%	ND	ND	ND	Alaska	1986–2010	Rudolph et al. 2013
S. pneumoniae	6.2%	3571	30%	56%	1.0%	ND	ND	ND	Finland	2002–2006	Siira et al. 2009
S. pneumoniae	21.5%	1007	41%	50%	2%	6%	ND	ND	Finland	2002	Rantala et al. 2005
S. pneumoniae	26%	863	54%	13%	31%	ND	ND	ND	Russia	2009–2013	Mayanskiy et al. 2014
S. pneumoniae	59.3%	555	47.7%	30.7%	21.6%	ND	ND	ND	Asia	1998–2001	Song et al. 2004
S. pneumoniae	9.5%	410	43.5%	56.5%	ND	ND	ND	ND	Chile	1997–1999	Palavecino et al. 2002
S. pneumoniae	67.7%	65	36.0%	18.0%	32%	ND	ND	ND	Lebanon	2008–2010	Taha et al. 2012
S. pneumoniae	2.4%–6.9%	3241	61.0%	33.1%	ND	ND	ND	ND	Columbia	1994–2008	Hidalgo et al. 2011
S. pneumoniae	26.4%	151	95.0%	5.0%	ND	ND	ND	ND	Turkey	1998–2002	Gulay et al. 2008
S. pneumoniae	21.4%	2045	27.8%	68.9%	4.1%	ND	ND	ND	Germany	2005–2006	Bley et al. 2011
Streptococcus pyogenes	8.2%	352	48.3%	31.0%	0%	ND	ND	ND	Germany		
S. pyogenes	12.5%	3893	28.1%	71.5%	ND[e]	ND	ND	ND	Serbia	2007–2008	Opavski et al. 2015
S. pyogenes	98.4%	127	100%	0.0%	0.0%	ND	ND	ND	China	2004–2011	Zhou et al. 2014
S. pyogenes	2.4%	739	29.7%	48.1%	22.8%	ND	ND	ND	United States (Utah)	2007–2008	Rowe et al. 2009
β-Hemolytic streptococci group											
A	1.9%	1625	57.7%	42.3%	0%	ND	ND	ND	Scotland	2000–2001	Amezaga and McKenzie 2006
B	4.3%	1233	88.1%	11.9%	0%	ND	ND	ND	Scotland	2000–2001	Amezaga and McKenzie 2006
C	3.8%	479	11.1%	66.7%	11.1%	ND	ND	ND	Scotland	2000–2001	Amezaga and McKenzie 2006
G	6.2%	1034	90.4%	9.6%	0%	ND	ND	ND	Scotland	2000–2001	Amezaga and McKenzie 2006

Continued

Table 2. *Continued*

Species	Percent macrolide resistance	Number of isolates	Macrolide-resistant mechanism						Country or region	Isolation year(s)	References
			erm[a]	*mef*(A)	*erm*[a] + *mef*(A)	Target[b]	*msr*(A)	*mph*(A)[c]			
β-Hemolytic streptococci group											
A	1.4%	219	66.7%	33.3%	0%	ND	ND	ND	The Netherlands	2005–2006	Buter et al. 2010
B	5.3%	562	93.3%	0%	0%	ND	ND	ND	The Netherlands	2005–2006	Buter et al. 2010
C	6.9%	58	100%	0%	0%	ND	ND	ND	The Netherlands	2005–2006	Buter et al. 2010
G	4.6%	237	45.5%	18.2%	9.1%	ND	ND	ND	The Netherlands	2005–2006	Buter et al. 2010
Group B streptococci	12.6%	143	77.8%	0%	22.2%	ND	0%	ND	Kuwait	2007	Boswihi et al. 2012
S. agalactiae	38.1%	93	75.7%	24.3%	ND	ND	ND	ND	France	2011–2012	Bergal et al. 2015
S. agalactiae	40%	226	97.8%	2.2%	0%	ND	ND	ND	Tunisia	2007–2009	Hraoui et al. 2012
Viridans group streptococci	27%	85	12.9%	12.9%	4.7%	ND	ND	ND	Turkey	1996–2004	Ergin et al. 2006
Viridans group streptococci	28.2%	85	66.7%	33.3%	ND	ND	ND	ND	Gran Canaria, Spain	2004–2006	Artiles Campelo et al. 2007
Streptococci	63.0%	413	22.6%	29.6%	0.4%	47.0%	ND	ND	Canada[f]	2006–2011	Thornton et al. 2015
S. aureus	49.7%	656	100%	ND	ND	ND	0%	ND	Greece	2003–2008	Vallianou et al. 2015
S. aureus	58.0%	106	95.1%	ND	ND	ND	1.6%	ND	Iran	2010–2011	Shahsavan et al. 2012
S. aureus	NR	97[e]	73.1%	ND	ND	ND	13.4%	ND	Poland	NR	Piatkowska et al. 2012
S. aureus	56.0%	100	41.1%	ND	ND	51.8%	7.1%	ND	Czech Republic[f]	2011–2013	Tkadlec et al. 2015
MRSA	72.8%	397	88.9%	ND	ND	ND	4.4%	ND	Turkey	2006–2008	Yildiz et al. 2014
MRSA	84.9%	265	83.1%	ND	ND	ND	35.6%	ND	Turkey	2003–2006	Gul et al. 2008
MRSA	79.1	158	89.6%	ND	ND	ND	10.4%	ND	Turkey	2012–2013	Aydeniz Ozansoy et al. 2015
MSSA	9.8%	246	75.0%	ND	ND	ND	25.0%	ND	Turkey	2012–2013	Aydeniz Ozansoy et al. 2015
S. aureus[d]	25.2%	111	82.1%	ND	ND	ND	75.0%	ND	Spain	1997–2006	Argudin et al. 2014
S. aureus	45.3%	203	51.1%	ND	ND	ND	34.8%	ND	Spain	2006–2007	Perez-Vazquez et al. 2009
S. aureus	34.1%	91	96.7%	ND	ND	ND	3.3%	ND	Italy	2005–2006	Gherardi et al. 2009
CoNS[g]	62.7%	89	47.1%	ND	ND	ND	52.9%	ND	Italy	2005–2006	Gherardi et al. 2009
CoNS	61.7%	494	73.1%	ND	ND	ND	27.5%	24.9%	Germany	2004–2006	Gatermann et al. 2007
S. epidermidis	57.4%	47	70.4%	ND	ND	ND	33.3%	ND	Mexico	2002–2004	Castro-Alarcon et al. 2011
S. epidermidis	62.8%	77	85.3%	0%	0%	ND	14.7%	ND	Tunisia	2002	Bouchami et al. 2007
S. epidermidis	90%	63	60%	ND	ND	ND	40.4%	ND	Poland	2009	Brzychczy-Wloch et al. 2013
S. haemolyticus	100%	28	7%	ND	ND	ND	92.9%	ND	Poland	2009	Brzychczy-Wloch et al. 2013

Continued

Table 2. *Continued*

Species	Percent macrolide resistance	Number of isolates	Macrolide-resistant mechanism						Country or region	Isolation year(s)	References
			erm[a]	*mef*(A)	*erm*[a] + *mef*(A)	Target[b]	*msr*(A)	*mph*(A)[c]			
Staphylococcus hominis	100%	55	67.5%	ND	ND	ND	18.2%	ND	Poland	ND	Szczuka et al. 2015
Staphylococcus saprophyticus	44.4%	72	15.6%	ND	ND	0.0%	81.3%	ND	France	2005–2009	Le Bouter et al. 2011
Haemophilus influenzae	25.5%	106	18.5%	29.6%	51.9%	0.0%	ND	ND	United States + Canada[f]	2007–2008	Roberts et al. 2011
Corynebacterium species	89.7%	140	87.8%	ND	ND	ND	ND	ND	Poland	2008–2011	Olender 2013
Mycoplasma genitalium	36.1%	155	ND	ND	ND	100%	ND	ND	Australia	2012–2013	Bissessor et al. 2015
M. genitalium	9.8%	297	ND	ND	ND	100%	ND	ND	Greenland	2008–2009	Gesink et al. 2012
M. genitalium	40%	1121	ND	ND	ND	100%	ND	ND	Denmark	2006–2010	Salado-Rasmussen and Jensen 2014
Mycoplasma pneumoniae	68.7%	67	ND	ND	ND	100%	ND	ND	China	2008–2009	Cao et al. 2010
M. pneumoniae	67.8%	1655	ND	ND	ND	100%	ND	ND	Japan	2008–2012	Kawai et al. 2013
M. pneumoniae	83%	53	ND	ND	ND	100%	ND	ND	China	2005–2008	Liu et al. 2009b
M. pneumoniae	40.4%	27	ND	ND	ND	100%	ND	ND	Korea	2011	Yoo et al. 2012
M. pneumoniae	88.0%	309	ND	ND	ND	100%	ND	ND	China	2008–2011	Zhao et al. 2013
M. pneumoniae	13.2%	91	ND	ND	ND	100%	ND	ND	United States	2012–2014	Zheng et al. 2015
M. pneumoniae	100%	71	ND	ND	ND	100%	ND	ND	China	2012–2014	Zhou et al. 2015
Ureaplasma urealyticum	80.6%	72	36.2%	ND	ND	ND	63.8%	ND	China	2008	Lu et al. 2010

Macrolide resistance is defined by resistance to erythromycin or clarithromycin; in the case of sexually transmitted pathogens, the species and macrolide resistance mechanisms were usually determined by real-time hydrolysis probe PCR targeting directly on urine samples/vaginal swabs. *M. pneumoniae* isolates were identified by colony morphology and PCR assay, real-time PCR melt curve analysis, and/or real-time PCR targeting of conserved genes and 23S rRNA mutations.

ND, Not determined; NR, not reported; MRSA, methicillin-resistant *S. aureus*; MSSA, methicillin-susceptible *S. aureus*.

[a]Generally *erm*(B) or *erm*(A), including subclass *erm*(TR), in streptococci; *erm*(A) *erm*(B), and/or *erm*(C) in staphylococci; *erm*(A), *erm*(B), *erm*(C), and/or *erm*(F) in *H. influenza*.

[b]Mutation(s) in ribosomal protein L4 or L22 or in domain V of 23S rRNA.

[c]*mph*(C) in Gatermann et al. (2007).

[d]Majority of isolates had multiple macrolide resistance genes and the most frequently found was *msr*(B)-*erm*(C).

[e]All erythromycin-resistant isolates characterized, no total number provided.

[f]Patients with cystic fibrosis.

[g]CoNS, coagulase-negative staphylococci.

chami et al. 2007) to 81% (France, Le Bouter et al. 2011) of coagulase-negative staphylococci (Gatermann et al. 2007; Perez-Vazquez et al. 2009; Le Bouter et al. 2011; Zmantar et al. 2011; Argudin et al. 2014).

Erythromycin, clarithromycin, and azithromycin are the therapeutic agents of choice for *M. pneumoniae* infections in children, and the first macrolide-resistant strain was isolated in Japan in 2000 (Okazaki et al. 2001). By 2003, 13% (13/76) of *M. pneumoniae* isolates in Japan were resistant to erythromycin (Matsuoka et al. 2004) with the majority ($n = 10$) associated with A2063G (A2058 *E. coli* numbering) and one each of A2063C, A2064G, and C2617G (C2611 *E. coli* numbering), with the latter only expressing weak resistant to erythromycin (MIC = 8 μg/ml) (Tables 1 and 2). By 2008, the prevalence had reached 30.6%. Prevalence increased from 2008 to 2012 (Kawai et al. 2013) with regional differences of macrolide resistance, varying from 50% to 93%, and with resistance rates higher in patients that had received macrolides before the surveillance study. The majority of the 561 isolates from 769 patients had mutations of A2063G or A2063T; less commonly, A2063C, A2064G, and C2617G were found. Rates of macrolide-resistant *M. pneumoniae* (MRMP) have exceeded 90% in Beijing, China (Zhao et al. 2013), with the majority of MRMP carrying the A2063G followed by A2064G and a single isolate with A2063T mutation. In Zhejiang, China, 100% of *M. pneumoniae* strains isolated from adults with community-acquired pneumonia carried the resistance determinant, A2063G mutation (Zhou et al. 2015).

Macrolides are often used for first-line therapies of *Ureaplasma urealyticum* infections. Interestingly, 80.6% of *U. urealyticum* in a 2008 study in China were macrolide-resistant (Lu et al. 2010). About 64% of the isolates harbored *msr*(A) ± *msr*(D), whereas 36% carried *erm*(B), perhaps reflecting that this species can host plasmids and transposons. For *M. genitalium*, another causative agent of sexually transmitted infections, only 23S rRNA mutations have been identified in macrolide-resistant isolates.

CONCLUDING REMARKS

Cryo-EM and X-ray crystallography have provided structural insights into how the ribosome interacts with and responds to small molecules like antibiotics. These studies help to explain how different target-based mutations or methylation of A2058 confer resistance, as well as provide an understanding into how regulation of *erm* methyltransferases and efflux genes has evolved. Along with the ribosome-macrolide X-ray structures, we now have a much clearer understanding of how macrolides inhibit protein synthesis (i.e., the link between the ribosomal tunnel and the PTC) and the data show us that macrolide action is specific, targeting a subset of proteins. Assays could be developed to ensure that the synthesis of certain vital proteins is impacted and/or to monitor the mechanism(s) of action, potentially enriching for compounds that promote frameshifting, for example. The route to total synthesis will allow the exploration of structure–activity relationships, overcoming any limitations of semisynthesis (see macrolide.com; Zhang et al. 2016) and potentially extending spectrum beyond the community-acquired respiratory pathogens. With our present understanding, perhaps it is prime time to rethink macrolide drug discovery and use the existing and expanding tool sets to find molecules with more refined, targeted actions.

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